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Award Number: DAMD17-00-1-0686

TITLE: Discovery of New Drugs that Target Peroxisomal Proliferator-Activated Receptor gamma (PPAR-gamma) for the Treatment of Breast Tumors

PRINCIPAL INVESTIGATOR: Dr. Dale Nagle

CONTRACTING ORGANIZATION: University of Mississippi
University, Mississippi 38677-1848

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PREPARED FOR: U.S. Army Medical Research and Materiel Command
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Kath Moore 1/8/02 _____

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12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The major goal of this project is to discover novel chemotherapeutic agents that act on a newly discovered molecular target in breast tumor cells. Recent studies have demonstrated that substances that activate the nuclear hormone receptor/transcription factor, Peroxisome Proliferator-Activated Receptors-gamma (PPAR-γ) can inhibit growth, cause terminal differentiation, and induce apoptosis in human breast tumor cells.

In vitro molecular mechanism-targeted pharmacological assays were developed and used to discover small (drug-like) PPAR-γ activators from chemically-rich marine organisms. Chemically unique marine oxylipins (structurally novel lipoxygenase metabolites of marine fatty acids) were shown to act as PPAR-γ ligands, transactivate PPAR-γ gene expression, induce cellular arrest, and cause cell death in MCF-7 breast tumor cells *in vitro*. Most currently know PPAR-γ activators are fatty acid metabolites or structurally related to the synthetic thiazoilidinedione (TZD) class of insulin sensitizers. This research has discovered that cyanobacteria and marine algae produce natural products, found nowhere else in nature, that activate PPAR-γ. These structurally unique marine natural products are chemically unrelated to any other class of known PPAR-γ activators. It is envisioned that these novel chemical prototypes can be used as "chemical ideas" to design new antitumor agents that function through the activation of PPAR-γ.

14. Subject Terms (keywords previously assigned to proposal abstract or terms which apply to this award)
Marine Natural Products, Peroxisome Proliferator-Activated Receptors, PPAR-γ oxylipin, eicosanoids, tumor-selective, gene expression, noncytotoxic

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Table of Contents

INTRODUCTION :

Objectives and Relevance to Breast Cancer: The major goal of this project is to discover new therapeutically useful chemotherapeutic agents that will act directly at the site of a newly discovered molecular target in breast tumor cells. The molecular target for this drug discovery program is the nuclear hormone receptor/transcription factor, known as Peroxisome Proliferator-Activated Receptor-gamma (PPAR-γ).

Supporting Rationale: Recent studies have established the role of PPAR-γ as central regulators of estrogen biosynthesis in human breast adipose tissue (1). Ligands (agonists) of PPAR-γ have been shown to inhibit growth, cause terminal differentiation, and induce apoptosis in human breast tumor cells (2,3). Eicosanoids (20-carbon oxidized and cyclized fatty acid metabolites) in human serum and tissues regulate blood glucose and lipid metabolism through activation of PPARs. We have developed a panel of in vitro molecular mechanism-targeted pharmacological assays for evaluating the activation and function of PPAR-γ. Our research and that of others has demonstrated that marine algae and invertebrates produce hundreds of chemically diverse unusual eicosanoids and related metabolites found nowhere else in nature (4). These molecules, now referred to as marine oxylipins, include lipoxygenase metabolites known to activate PPAR-γ, as well as structural analogs of endogenous human PPAR-γ ligands (hydroxy-eicosanoids and prostaglandins) that specifically regulate the function of each PPAR-γ. However, many of these marine oxylipins contain unique molecular substitutions and stereochemical features that distinguish them from all known mammalian fatty acid metabolites.

Hypothesis: We hypothesize that marine oxylipins are pharmacological analogs of human eicosanoid PPAR-γ regulators that will cause terminal differentiation of breast tumor cells and can be used to develop new drugs to treat breast cancer. The specific aims of this proposal are: 1. Evaluate *in vitro* activity of the extracts for the ability to directly regulate PPAR-γ; 2. Isolate and characterize the active constituents.

BODY:

As a Concept Award a Statement of Work was not filed for this research. However, we have enclosed the "General Plan" outlined for the proposed work. The complete general plan from the original proposal is as follows: This project represents a translational scientific approach that integrates marine natural products chemistry and biology, with modern receptor pharmacology and state-of art molecular biology to develop a breast cancer drug discovery program. To accomplish this, an interdisciplinary collaborative research team of scientists has been established. The P.I. has assembled a repository of several hundred extracts and purified compounds from marine algae and invertebrates collected throughout the world. These samples include a percentage of oxylipin-containing and natural product-rich specimens to ensure a high likelihood of success, within one year. This project will examine the activity of marine extracts on activation of human PPAR-γ. Using cell-based luciferase reporter gene assays, we plan to examine the concentration-dependent activation of PPAR-γ using both estrogen-dependent (MCF-7) and estrogen-independent (MDA-MB-231) human breast tumor cells transiently transfected with PPAR-γ receptor and reporter plasmids by techniques described in recent publications. These cell-based assays have been adapted to 96-well microtiter plates and provide us with high throughput assays for this project. Standard activators of PPAR-γ (BRL 49,653) will be used in each assay. The high-throughput versions of PPAR-γ assays will be developed and supervised by both the P.I. and the Co-P.I. Activators of PPAR-γ will be evaluated for cytostatic and cytotoxic activity against human breast tumor cell lines (National Center for Natural Products Research Cancer Program). PPAR-γ activation activity will guide the chromatographic separation of active extracts. Primary bioassays will be measured throughout all stages of fractionation and chromatographic separation of marine natural products. Molecular structures of active natural products will be elucidated through a combination of modern spectroscopic and spectrometric methods including Multi-Dimensional Nuclear Magnetic Resonance Spectroscopy (NMR) and High Resolution Mass Spectrometry. The P.I. has ten years of experience with these techniques. Successful completion of the proposed work will yield new prototype lead compounds that target PPAR-γ and will serve as templates for new classes of drugs to treat breast cancer.

Experimental Results

(A)

The initial phase of this project involved developing *in vitro* molecular mechanism-targeted high-throughput assays for PPAR-γ activators in two human breast tumor cell lines (MCF-7 and MDA-MB-231*)*. The results are described below with reference to specific data shown in the following appended figures:

Appendix **Figure 1**) Activation of transcription from the PPAR-γ response element (PPRE-aP2 reporter) in the estrogen receptor negative and highly malignant MDA-MB-231 cells requires exogenous PPAR-γ (from co-transfected rat PPAR-γ expression vector). Fetal calf serum (10%) showed 2.5-fold induction after 6 hr incubation.

Appendix **Figures 2a-c**) Activation of transcription from the PPAR-γ response element (PPRE-aP2 reporter) was examined in MCF-7 and MDA-MB-231 cells transiently transfected with both

reporter (PPRE-aP2) and expression vector (rat PPARγ), using four reported PPAR activators at different concentrations for different amount of incubation time (6, 24, 48 hrs). **a**) At 6 hrs little to no significant activation was observed following treatment with any PPAR activator. Neither cell line showed more than a 50% increase in activation, relative to that of the control. **b**) At 24 hrs only the known PPAR-γ activator troglitazone began to produce a greater than 50% increase in activation response (in MCF-7 cells), relative to control. **c**) At 48 hrs indomethacin produced only a very week activation response, ciglitazone and troglitazone moderately activated the PPRE-aP2 reporter in both cell lines, and 15-deoxy-Δ12,14-PGJ_2 strongly activated the reporter (10-fold induction at 10 μM in MCF-7 cells).

Appendix **Figures 3a-c**) Since the retinoid X receptor (RXR) heterodimerizes with PPAR-γ prior to activation of gene expression, it was envisioned that optimal activation of the PPRE-aP2 reporter should involve a synergistic combination of PPAR-γ activator and the RXR ligand 9-*cis*-retinoic acid. In order to establish high-throughput assays for activators of PPAR-γ, the optimal conditions for the synergistic activation of transcription from a PPAR-γ response element in the breast tumor cell lines (both MCF-7 and MDA-MB-231) were determined experimentally. **a**) At 6 hrs no combination produced any synergistic activation response. **b**) At 24 hrs both ciglitazone (10 μM) and troglitazone (10 μM) activated the PPRE-aP2 reporter in MCF-7 cells. No significant activation was observed in MCF-7 cells with any combination. However, 9-*cis*-retinoic acid significantly enhanced PPRE-aP2 reporter activation when combined with troglitazone or 15-deoxy-Δ12,14-PGJ_2. **c**) At 48 hrs 9-*cis*-retinoic acid (10 μM) dramatically enhanced the level of PPRE-aP2 activation induced by 15-deoxy-Δ12,14-PGJ_2 (10 μM) in both MCF-7 and MDA-MB-231 cells.

Appendix **Figure 4**) The relationship between activation of the PPRE-aP2 reporter and cell viability was examined in MDA-MB-231 cells following a 24 hr treatment with PPAR-γ activators, 9-*cis*-retinoic acid, and combinations of both. PPRE-aP2 activation was shown to directly correlate with a decrease in MDA-MB-231 viability.

(B)

The breast tumor cell line MCF-7 was shown to be well suited for use in a cell-based reporter gene assay for the detection of PPAR-γ activators (figures 2 and 3). According to published research by other groups, MCF-7 cells express PPAR-γ protein, while no endogenous PPAR-γ protein was detected in MDA-MB-231 cells. Our research has been focused on identifying activators of PPAR-γ in MCF-7 cells transiently transfected with the PPRE-aP2-luc reporter. The results are shown in the following figures.

Appendix **Figure 5**) At 24 hrs ciglitazone (10 μM) and 9-*cis*-retinoic acid (10 μM) each moderately activate the PPRE-aP2 reporter in MCF-7 cells. However, at 24 hrs 15-deoxy-Δ12,14-PGJ_2 (10 μM) does not significantly activate the reporter.

Appendix **Figure 6**) At 48 hrs the PPRE-aP2 activation induced by ciglitazone and 9-*cis*-retinoic acid is dwarfed by the dramatic activation (10-fold) of the PPAR-γ response element produced by 15-deoxy-Δ12,14-PGJ_2 (10 μM).

Appendix **Figures 7a-b**) Dose-response data (in MCF-7 cells) was obtained for PPRE-aP2 reporter activation induced by ciglitazone and 15-deoxy-Δ12,14-PGJ_2 at concentrations of 1.0 and 10 μM (with and without 10 μM 9-*cis*-retinoic acid) were obtained at 24 and 48 hrs. **a**) At 24 hrs only 15-deoxy-Δ12,14-PGJ_2 (10 μM) combined with 9-*cis*-retinoic acid significantly activated the PPAR-γ reporter (3.5-fold). **b**) At 48 hrs 15-deoxy-Δ12,14-PGJ_2 (10 μM) activated the PPRE-aP2 reporter (10-fold), without the addition of any 9-*cis*-retinoic acid . However, when 9-*cis*-retinoic acid (10 μM) was combined with 15-deoxy-Δ12,14-PGJ_2 (10 μM) an even greater activation of the PPAR-γ reporter was observed. The synergistic action of substrates induced approximately a 30-fold increase in reporter activity over that observed in the control.

Appendix **Figures 8a-d**) The relationship between PPAR-γ activation and tumor cell viability was studied in a set of experiments that examined the effects of the synthetic PPAR-γ activators, the natural ligand 15-deoxy-Δ12,14-PGJ_2, and various combinations of retinoids (9-*cis*-retinoic acid, 13-*cis*-retinoic acid, and all-*trans*-retinoic acid). Tumor cell viability was monitored using the Neutral Red Method. It has been shown that MCF-7 cells endogenously express PPAR-γ, this series of experiments were also designed to test the hypothesis that exogenous PPAR-γ receptor is not required for the activation of PPRE-aP2-luc reporter in MCF-7 cells. **a**) In 48 hr incubation experiments, ciglitazone concentrations up to 10 μM produced no significant activation of the PPRE-aP2 reporter in MCF-7 cells. Reporter activation was detected when this same experiment was performed in the presence of exogenous receptor (from co-transfected rPPAR-γ expression vector). The prostaglandin 15-deoxy-Δ12,14-PGJ_2 (10 μM) induced a strong activation of the reporter (12-fold) without the addition of the rPPAR-γ expression vector. In both cases, activation of the PPRE-aP2 reporter by 15-deoxy-Δ12,14-PGJ_2 (10 μM) was associated with a corresponding decrease in MCF-7 cell viability. **b**) While the retinoids (9-*cis*-retinoic acid, all-*trans*-retinoic acid, and 13-*cis*-retinoic acid) produce a weak increase in PPRE-aP2 reporter activity at 10 μM, they did not significatly effect MCF-7 cell viability (with or without exogenously introduced rPPAR-γ expression vector). **c**) Combinations of PPAR-γ activators and retinoids were examined for the ability to activate the PPRE-aP2 reporter and decrease cell viability. The combination of 15-deoxy-Δ12,14-PGJ_2 (10 μM) with either 9-*cis*-retinoic acid (10 μM) or 13-*cis*-retinoic acid (10 μM) consistently produced the strongest reporter activity and a substantial drop in MCF-7 viability. **d**) The combination of 15-deoxy-Δ12,14-PGJ_2 (10 μM) with all-*trans*-retinoic acid (10 μM) produced a similar response as observed with combinations using *cis*-retinoic acids in the previous figures. Strong PPRE-aP2 reporter activation was associated with a substantial drop in MCF-7 viability.

(C)

Once a sensitive and reproducible high-throughput assay was established, natural products produced by plants and marine organisms could be evaluated for their ability to activate the PPRE-aP2 reporter in the breast tumor cell lines. A panel of more than 100 pure chemically unique and structurally diverse secondary metabolites produced by marine algae and invertebrates were examined for the ability to activate PPAR-γ in MCF-7 cells. Most natural products examined show no ability to induce PPAR-γ reporter activation. However, we have discovered that cyanobacteria and marine algae produce at least four chemical classes of structurally unique

natural products that activate PPAR-γ. Experiments to determine ED_{50} values are currently in progress. Many of these marine natural products are chemically unrelated to any other known class of PPAR-γ activators. The structures and the concentrations of each natural product that produced significant reporter activation in preliminary experiments are presented in the following figures:

Appendix **Figure 9**) Specific types of structurally novel marine fatty acid metabolites (oxylipins) were shown to act as PPAR-γ ligands, transactivate PPAR-γ, induce cellular arrest, cause cell death in MCF-7 breast tumor cells *in vitro*. These include hydroperoxide-lyase products of lipid peroxides (Cymathere aldehyde), epoxidized prostaglandin analogs (ecklonialactones), omega-3 hydroxy fatty acid metabolites, and cyclized metabolites. None of these unusual marine oxylipins were previously known to act as activators of PPAR-γ.

Appendix **Figure 10**) Lipopeptides produced by marine cyanobacteria activate the PPRE-aP2 reporter in MCF-7 cells. Some of these metabolites appear to function as partial agonists and only weakly activate the PPAR-γ reporter. No lipopeptide natural products have previously been demonstrated to act as activators of PPAR-γ.

Appendix **Figure 11**) New antitumor agents that function through the activation of PPAR-γ can be designed from these newly discovered structural prototypes. Unusual marine algal biphenyl metabolites such as avrainvilleol are structurally related to synthetic phenolic PPAR-γ activators. However, styrylchromones (hormothamnione diacetate) and brominated prenylated hyroquinones (cymopol) are distinctly different from any other known phenolic activators of PPAR-γ.

KEY RESEARCH ACCOMPLISHMENTS:

Developed *in vitro* molecular mechanism-targeted high-throughput assays for PPAR-γ activators in two human breast tumor cell lines

Established optimal conditions for the synergistic activation of transcription from a PPAR-γ response element in breast tumor cell-based reporter gene assays

Demonstrated the utility of an MCF-7 breast tumor cell-based reporter gene assay for the detection of PPAR-γ activators, using a panel of synthetic ligands and natural eicosanoids

More than 100 pure chemically unique secondary metabolites produced by marine algae and invertebrates were examined for the ability to activate PPAR-γ in MCF-7 cells

Specific types of structurally novel marine fatty acid metabolites (oxylipins) were shown to act as PPAR-γ ligands, transactivate PPAR-γ, induce cellular arrest, cause cell death in MCF-7 breast tumor cells *in vitro*

Discovered that cyanobacteria and marine algae produce at least four chemical classes of structurally unique natural products that activate PPAR-γ. Many of these marine natural products are chemically unrelated to any other known class of PPAR-γ activators.

New antitumor agents that function through the activation of PPAR-γ can be designed from these newly discovered structural prototypes

REPORTABLE OUTCOMES:

While this project was a short-term exploratory Concept Award and the research is currently still in progress, reportable outcomes will be developed shortly. This project has successfully discovered new low molecular weight activators of PPAR-γ that provide strong preliminary support for expanding this research in forthcoming natural product-based breast cancer research grant proposals. At least four scientific publications are planned from this research. These findings will be first presented at upcoming 2002 Gordon Research Conference on Marine Natural Products in the form of an invited seminar (D.G. Nagle) and at least one other presentation by members of our research group (Feb. 2002, Ventura, CA). The seminar title is: "Targeting gene expression for the discovery of new tumor-selective chemotherapeutic agents." This project has contributed to the dissertation research of two graduate Ph.D. candidates.

CONCLUSIONS:

Recent research greatly supports the idea of using low molecular weight activators of the nuclear hormone receptor/transcription factor, known as Peroxisome Proliferator-Activated Receptor-gamma (PPAR-γ), as new relatively tumor-specific chemotherapeutic agents. Several classes of chemicals, including synthetic thiolizadendione oral antidiabetic agents, are known to activate PPAR-γ. Most known PPAR-γ activators are synthetic analogs of the thiolizadendiones or lipid, such as 15-deoxy-Δ12,14-PGJ_2. Currently, little is known in regard to the overall suitability and therapeutic potential of these known activators for the actual treatment of neoplastic disease. The discovery of several new classes of previously unknown marine natural product-based PPAR-γ activators, provides . The findings of this research suggest that natural products, particularly marine natural products, can provide novel chemical structure prototypes that can lead the development of new chemotherapeutic agents that induce breast tumor differentiation and promote tumor cell death, through the activation of PPAR-γ.

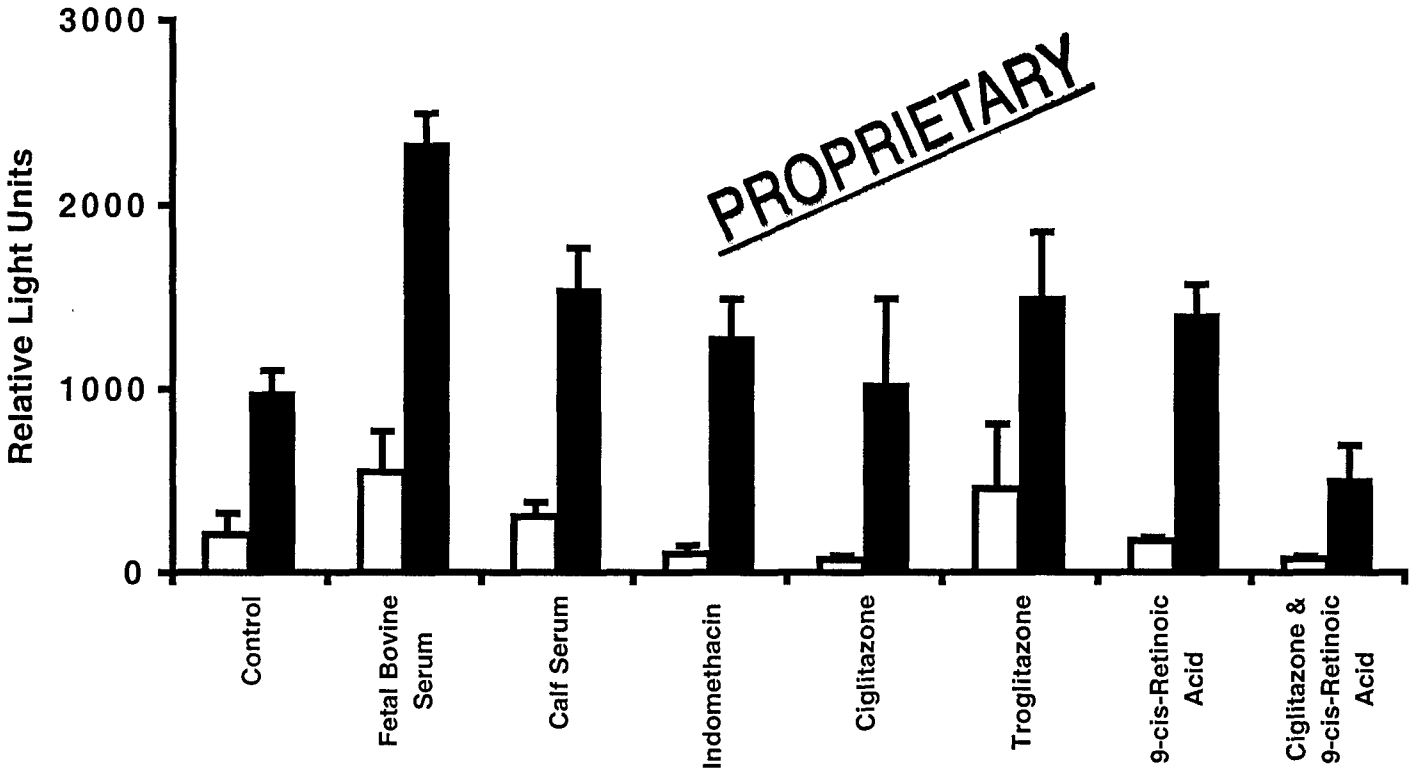
REFERENCES: NA

APPENDICES:

Figures 1-11 attached

Figure 1

Activation of Transcription from PPARgamma Response Element (PPRE-aP2) in MDA-MB-231 Cells



Exponentially grown MDA-MB-231 cells were transiently transfected with reporter alone (PPRE-aP2-luc, open bar) or a mix containing reporter and expression plasmid encoding rat PPARgamma (PPRE-aP2-luc/rPPARgamma), plated at the density of 20,000/well in culture medium containing 10% fetal calf serum. After cells attached, the medium was replaced by serum-free medium and incubated overnoight. The inducing agents were added and incubated for 6 hrs, cells lysed and luciferase activity analyzed. The final concentrations of the inducing agents are 10% for FBS and CS, 100 uM for indomethacin, and 10 uM for all the others. Data are presented as average plus standard error (n = 3).

Figure 2a Dose Response of PPARgamma Ligands in Activating Transcription from PPRE-aP2-luc Reporter (6 hrs)

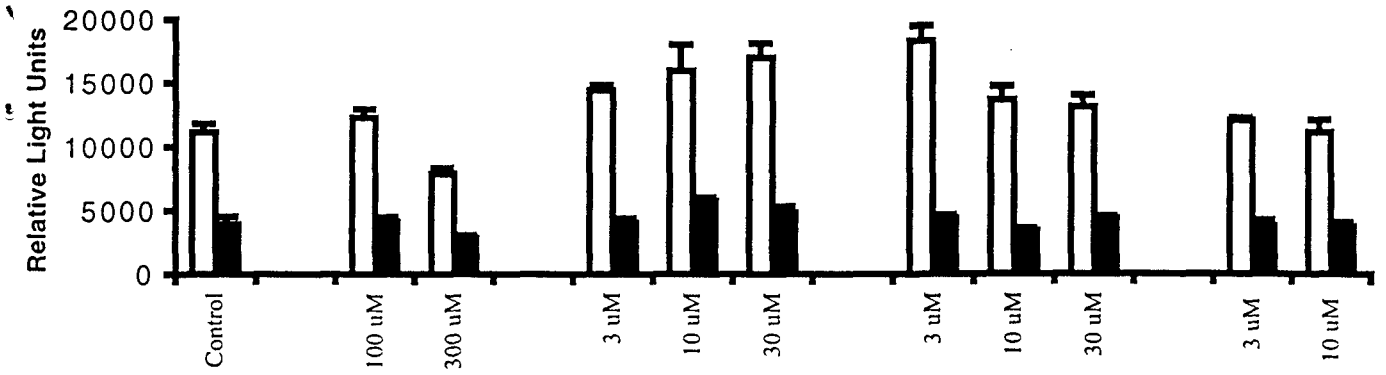


Figure 2b Dose Response of PPARgamma Ligands in Activating Transcription from PPRE-aP2-luc Reporter (24 hrs)

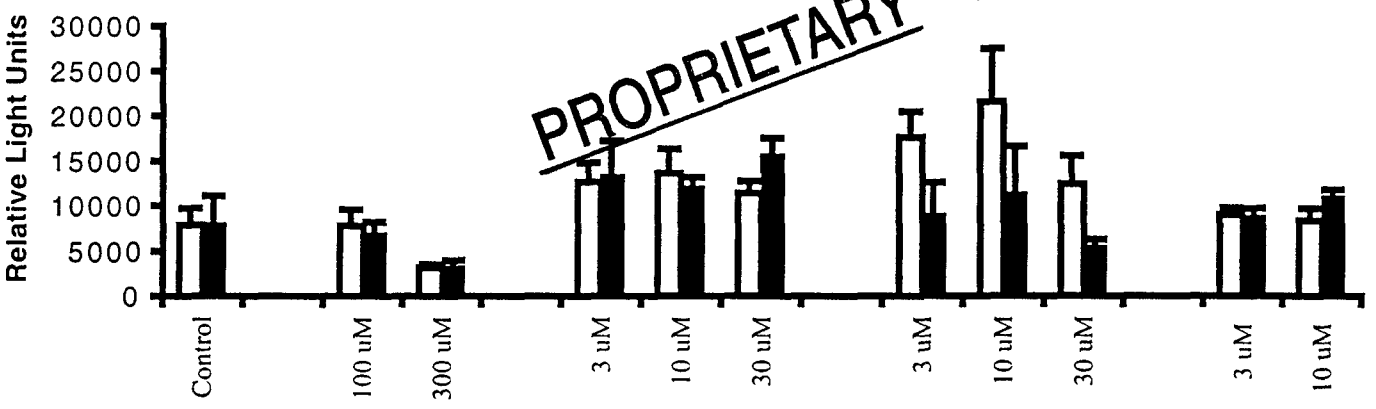
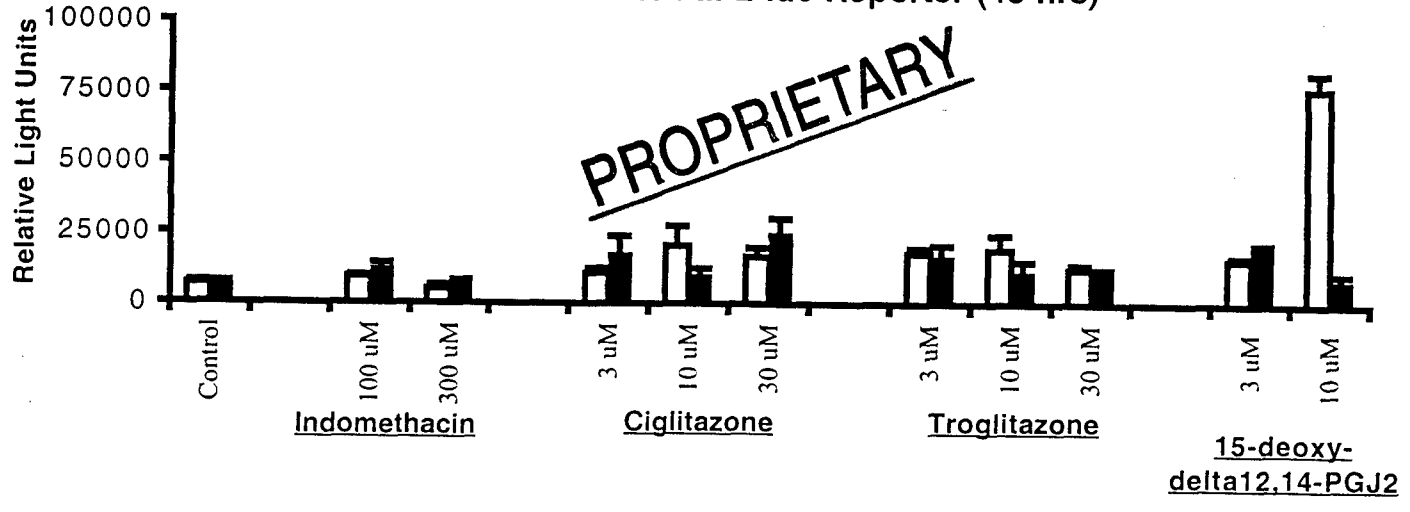


Figure 2c Dose Response of PPARgamma Ligands in Activating Transcription from PPRE-aP2-luc Reporter (48 hrs)



Exponentially grown MCF-7 and MDA-MB-231 cells were transiently transfected with a mix containing PPRE-aP2 reporter and expression vector encoding rat PPARgamma (PPRE-aP2-luc/rPPARgamma), plated at the density of 40,000/well in 100 ul culture medium containing 10% fetal calf serum into 96-well plates. After incubation at 37 degree for twenty hours, the inducing agents were added in 100 ul serum-free medium to achieve the desired concentrations, and incubated for another 6, 24, and 48 hrs. Cells lysed and luciferase activity analyzed. Data are presented as average plus standard error (n = 3), the open bars represent data obtained in MCF-7 cells, and the solid bars MDA-MB-231 cells.

Figure 3a Activation of PPRE-aP2-luc Reporter by PPARgamma Ligands in the Presence and Absence of the RXR Ligand 9-cis-Retinoic Acid (6 hrs)

Figure 3b Activation of PPRE-aP2-luc Reporter by PPARgamma Ligands in the Presence and Absence of RXR Ligand 9-cis-Retinoic Acid (24 hrs)

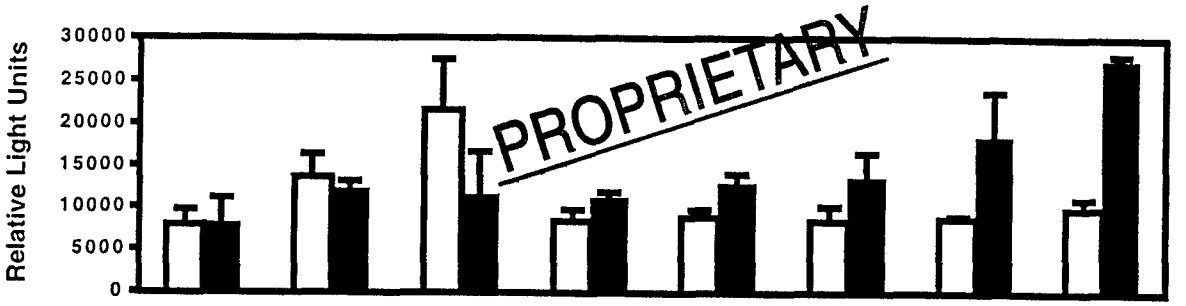
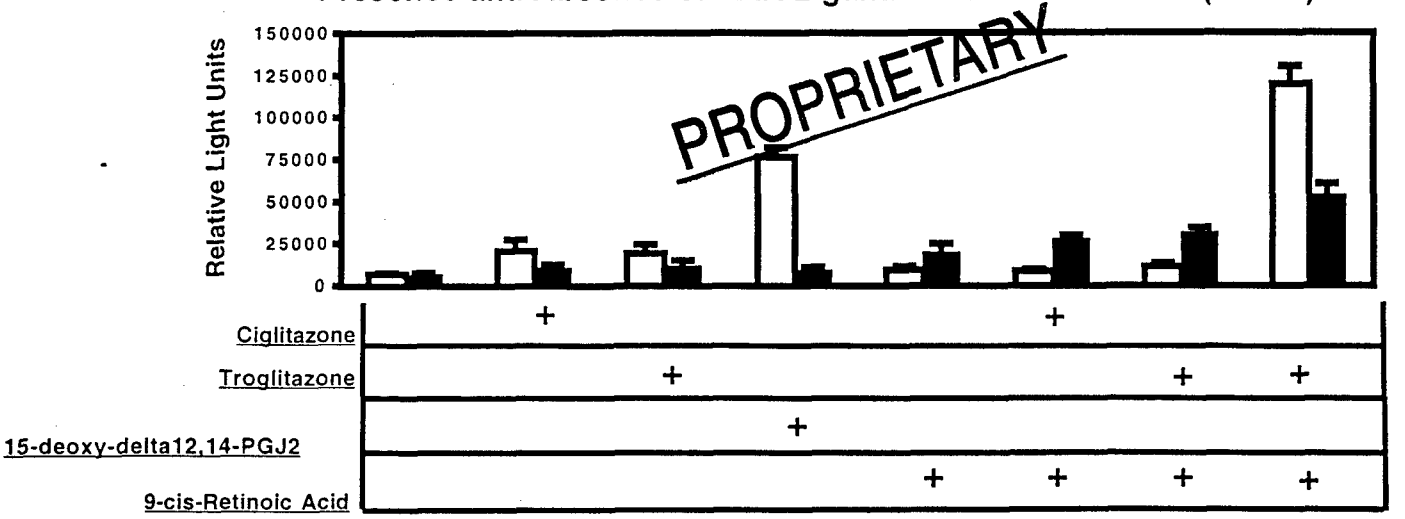


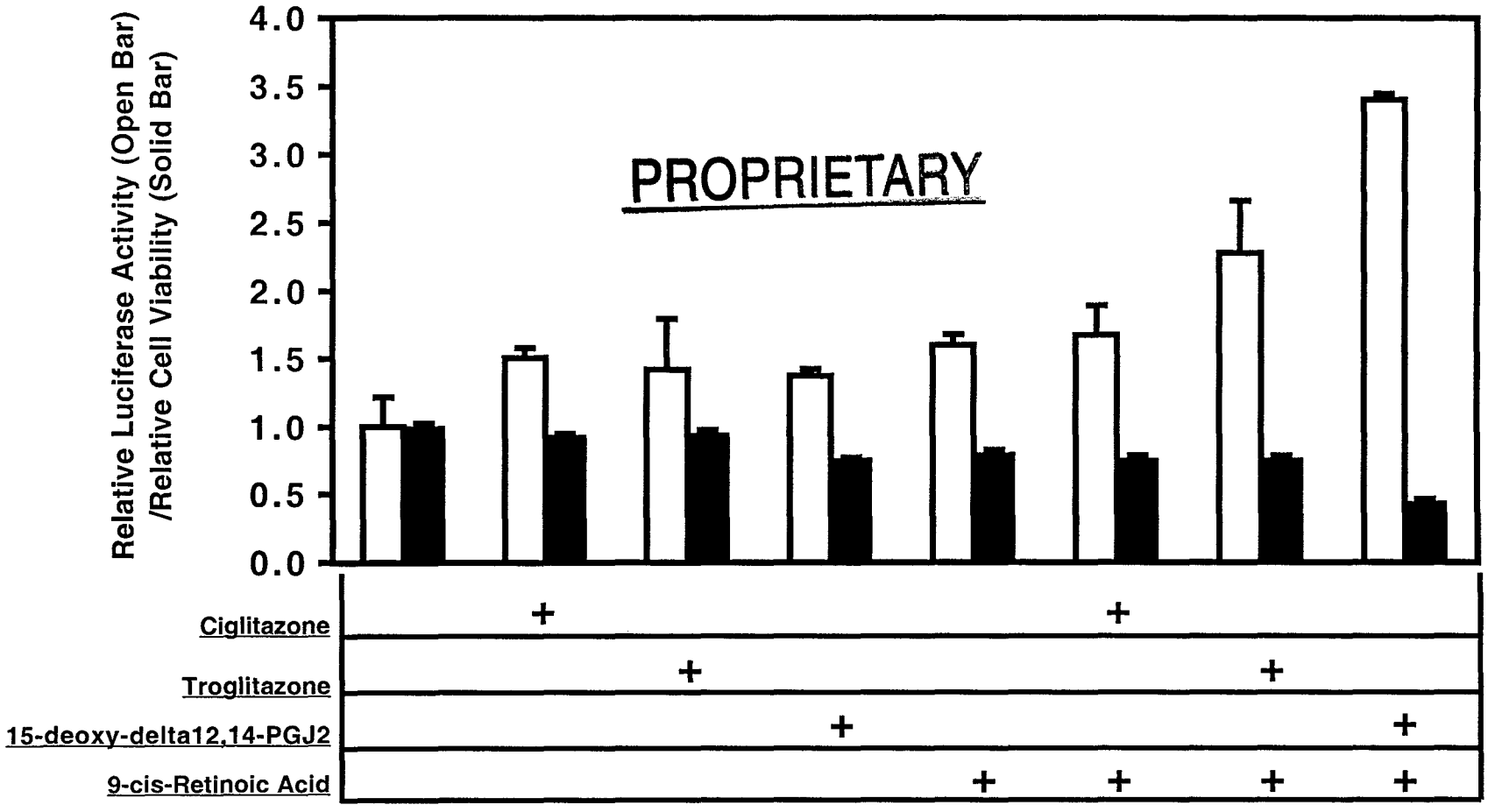
Figure 3c Activation of PPRE-aP2-luc Reporter by PPARgamma Ligands in the Presence and Absence of RXR Ligand 9-cis-Retinoic Acid (48 hrs)



Exponentially grown MCF-7 and MDA-MB-231 cells were transiently transfected with a mix containing PPRE-aP2 reporter and expression vector encoding rat PPARgamma (PPRE-aP2-luc/rPPARgamma), plated at the density of 40,000/well (6hrs induction) and 20,000/well (24 and 48 hrs induction) in 100 ul culture medium containing 10% fetal calf serum into 96-well plates. After incubation at 37 degree for twenty hours, the inducing agents were added in 100 ul serum-free medium to achieve 10 uM final concentration, and incubated for another 6, 24, and 48 hrs. Cells lysed and luciferase activity analyzed. Data are presented as average plus standard error (n = 3), the open bars represent data obtained in MCF-7 cells, and the solid bars MDA-MB-231 cells.

Figure 4

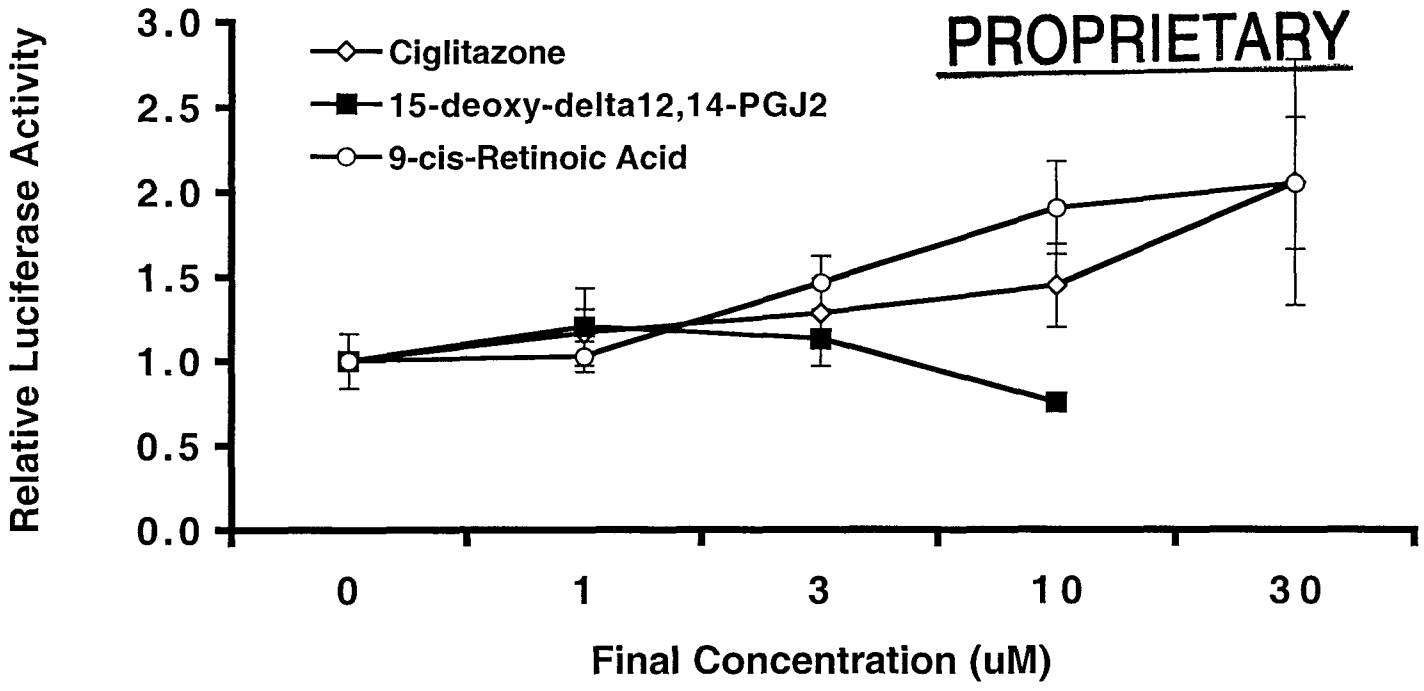
Activation of PPRE-aP2-luc Reporter and Cell Viability in MDA-MB-231 Cells (24 hrs)



Exponentially MDA-MB-231 cells were transiently transfected with a mix containing PPRE-aP2 reporter and expression vector encoding rat PPARgamma (PPRE-aP2-luc/rPPARgamma), plated at the density of 20,000/well in 100 ul culture medium containing 10% fetal calf serum into 96-well plates. After incubation at 37 degree for twenty hours, the inducing agents were added in 100 ul serum-free medium to achieve 10 uM final concentration, and incubated for another 24 hrs. Cells lysed and luciferase activity analyzed. Cell viability measured by Neutral Red Method. Data are converted to fold of control and presented as average plus standard error (n = 3), the open bars represent luciferase acitvity, and the solid bars cell viability.

Figure 5

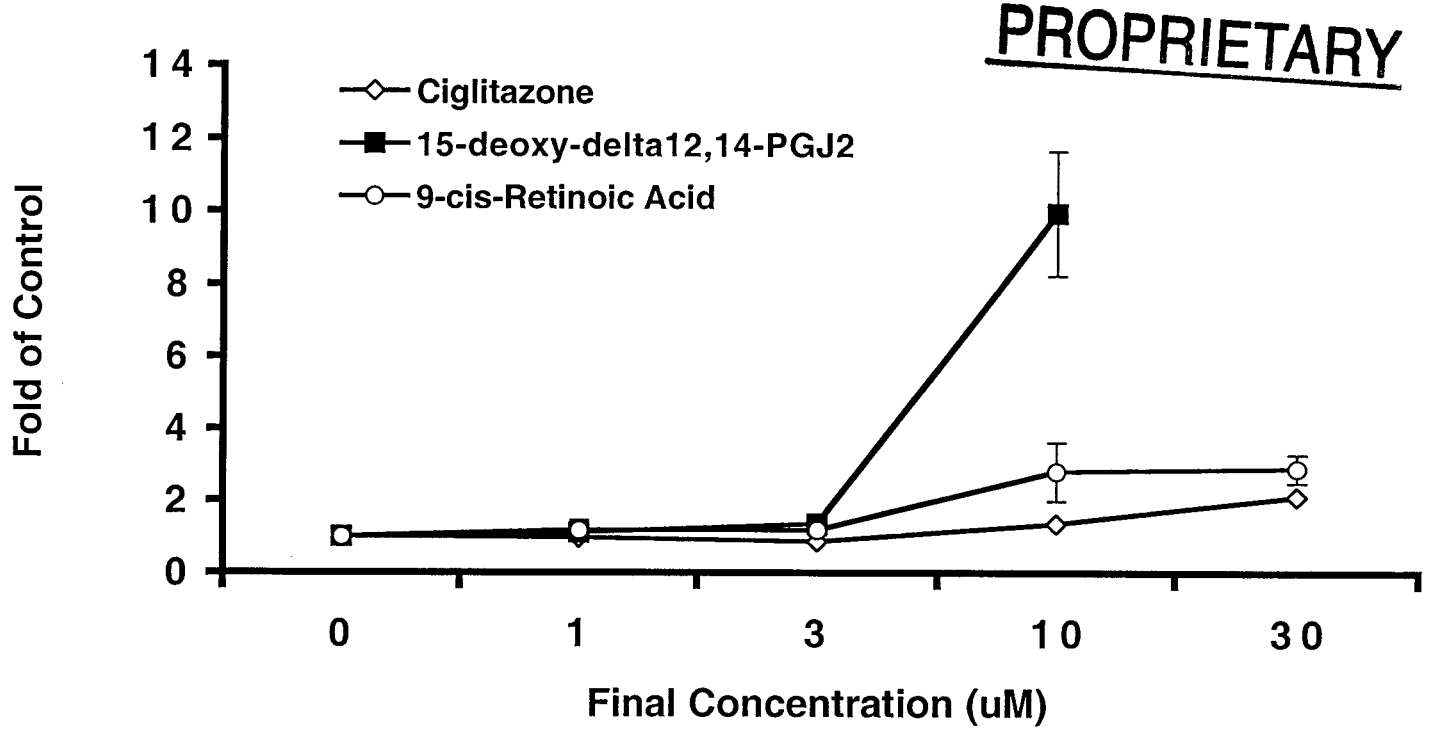
Dose-Response of PPARgamma and RXR Ligands in Activating PPRE-aP2-luc Reporter in MCF-7 Cells (24 hrs)



Exponentially grown MCF-7 cells were transiently transfected with PPRE-aP2 reporter(alone), plated at the density of 20,000/well in 100 ul culture medium containing 10% fetal calf serum into 96-well plates. After incubation at 37 degree for twenty hours, the inducing agents were added in 100 ul serum-free medium to achieve the desired concentrations, and incubated for another 24 hrs. Cells lysed and luciferase activity analyzed. Data are presented as average fold of control plus/minus standard error (n = 3).

Figure 6

Dose-Response of PPARgamma and RXR Ligands in Activating PPRE-aP2-luc Reporter in MCF-7 Cells (48 hrs)



Exponentially grown MCF-7 cells were transiently transfected with PPRE-aP2 reporter(alone), plated at the density of 20,000/well in 100 ul culture medium containing 10% fetal calf serum into 96-well plates. After incubation at 37 degree for twenty hours, the inducing agents were added in 100 ul serum-free medium to achieve the desired concentrations, and incubated for another 48 hrs. Cells lysed and luciferase activity analyzed. Data are presented as average fold of control plus/minus standard error (n = 3).

Figure 7a

Activation of PPRE-aP2-luc Reporter by PPARgamma Ligands in the Presence and Absence of RXR Ligand 9-cis-Retinoic Acid in MCF-7

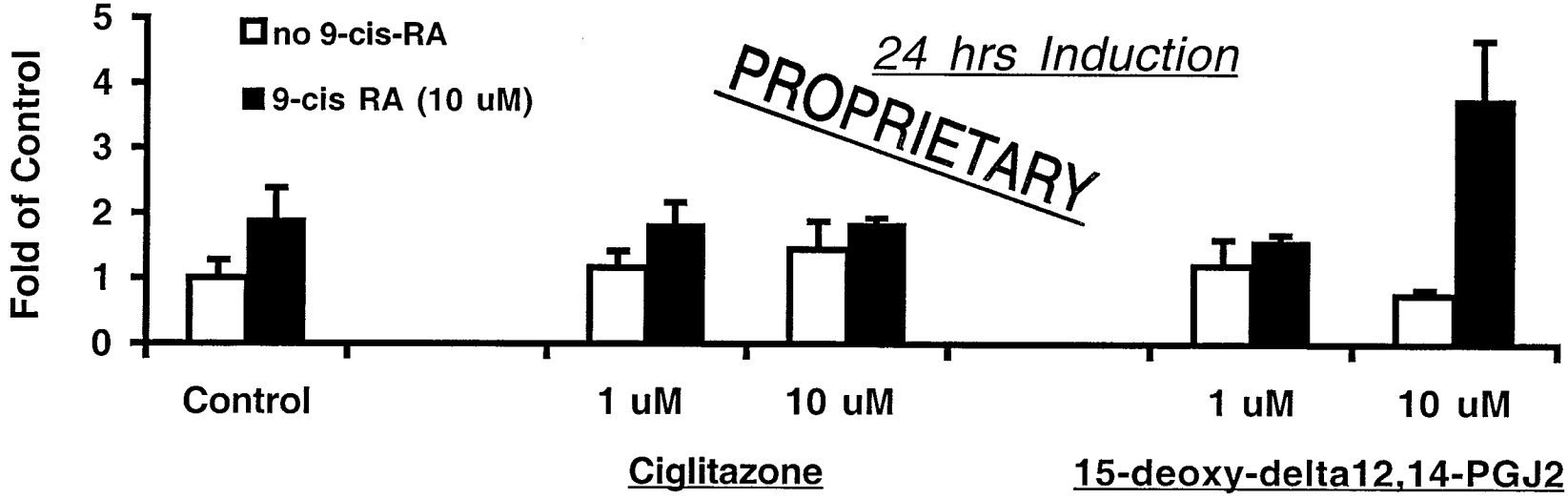
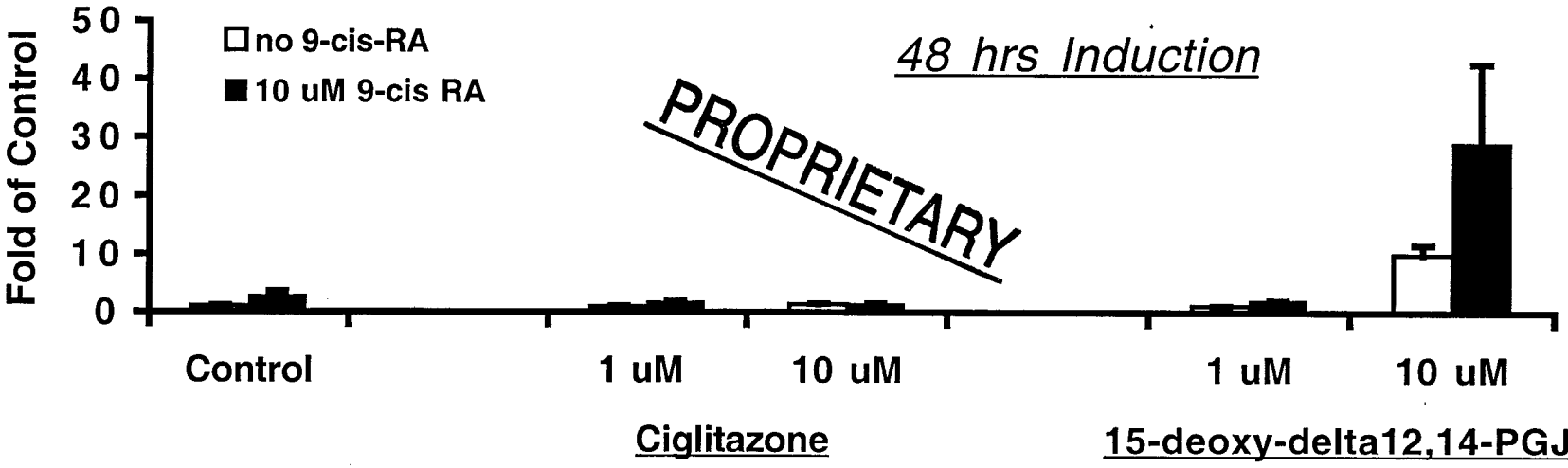


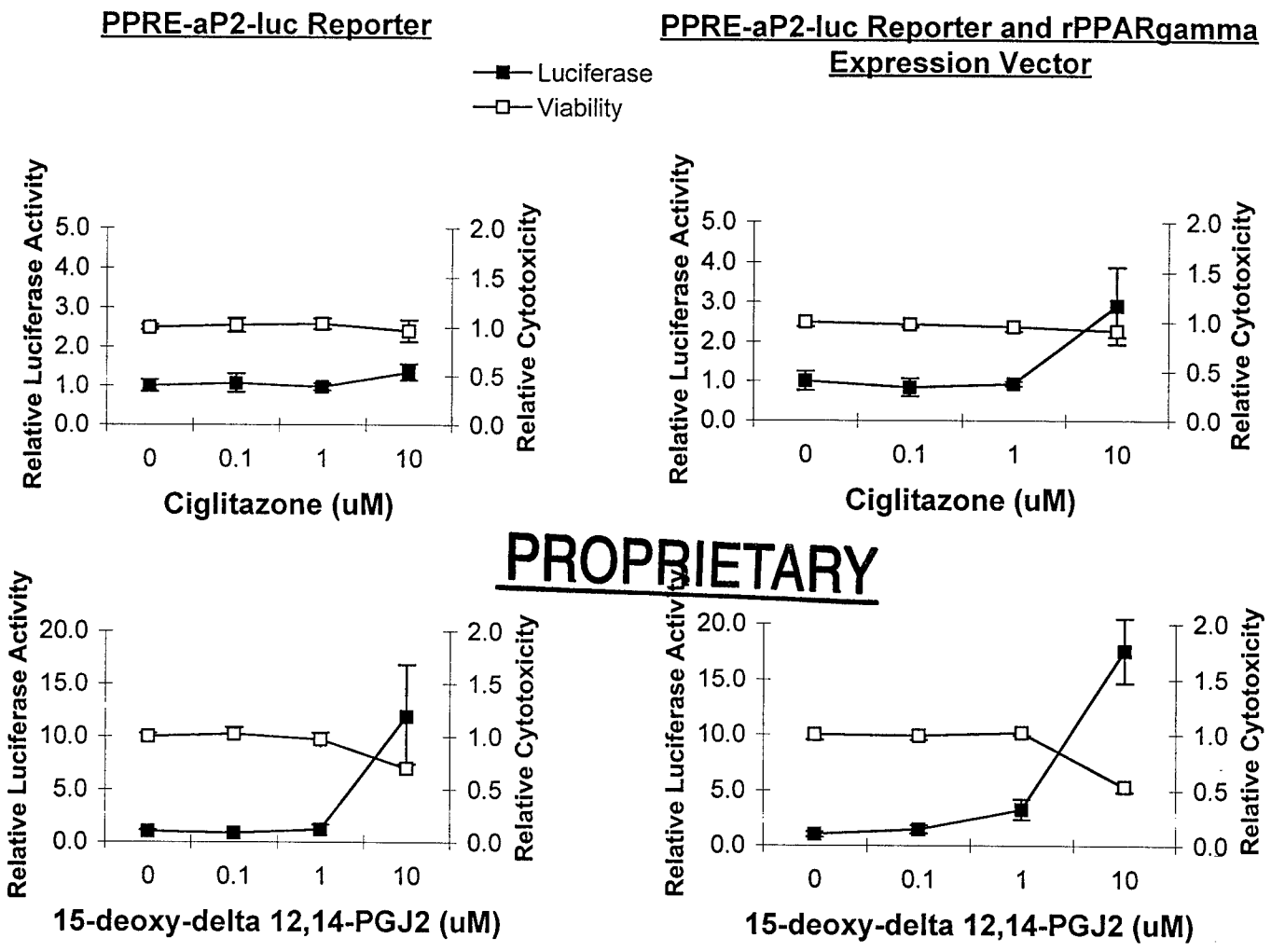
Figure 7b



Exponentially grown MCF-7 cells were transiently transfected with PPRE-aP2-luc reporter, plated at the density of 20,000/well in 100 ul culture medium containing 10% fetal calf serum into 96-well plates. After incubation at 37 degree for twenty hours, the inducing agents were added in 100 ul serum-free medium to achieve desired concentration, and incubated for another 24 and 48 hrs. Cells lysed and luciferase activity analyzed. Data are presented as average fold of control plus standard error (n = 3).

Figure 8a

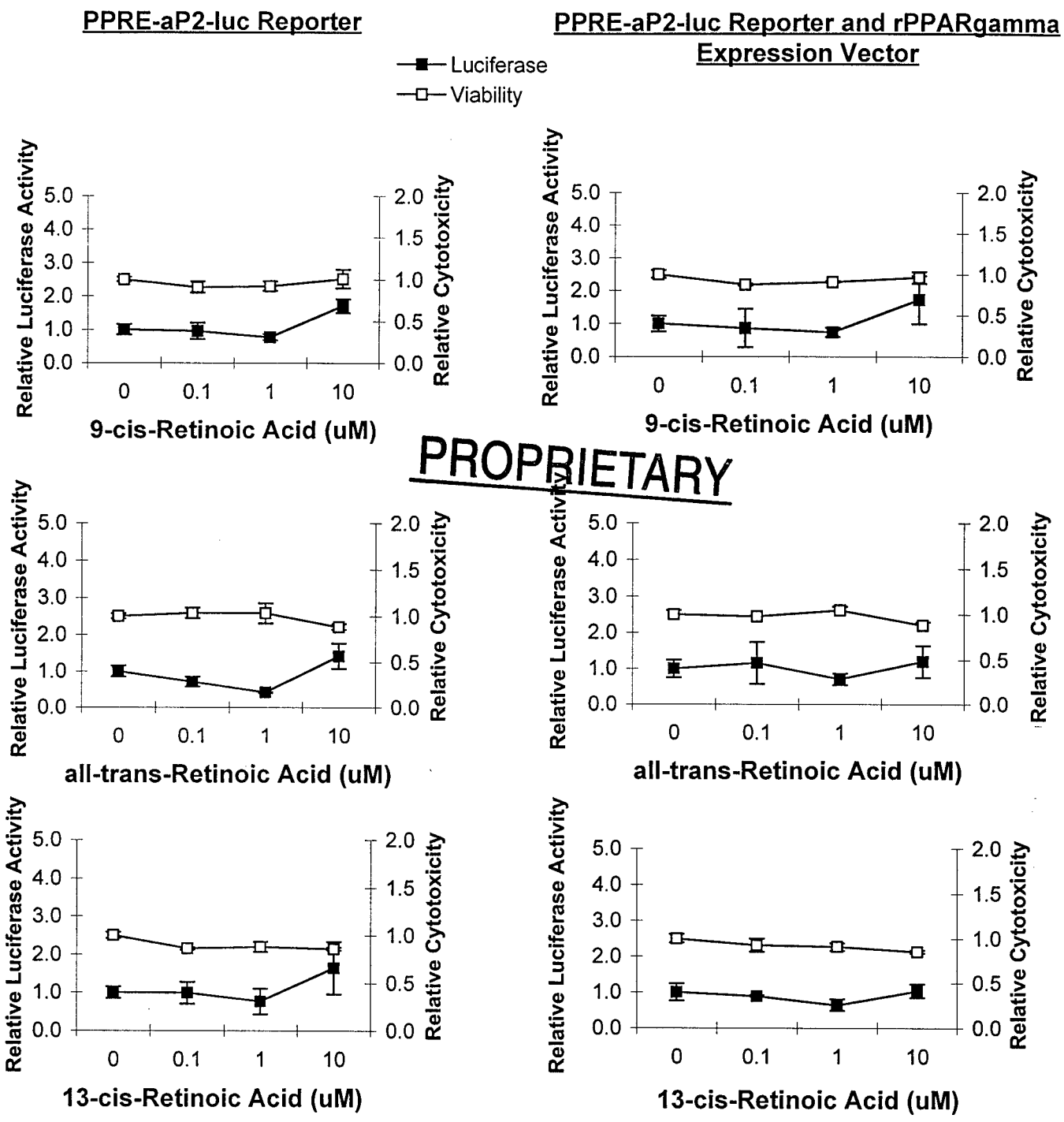
Dose-Response of PPARgamma Ligands in Activating PPRE-aP2 Reporter and Cell Viability in MCF-7 Cells (48 hrs)



Exponentially grown MCF-7 cells were transiently transfected with either PPRE-aP2-luc reporter alone (left panel) or a mix of PPRE-aP2-luc reporter and rat PPARgamma expression vector (right panel), plated at the density of 20,000/well in 100 ul culture medium containing 10% fetal calf serum into 96-well plates. After incubation at 37 degree for twenty hours, the inducing agents were added in 100 ul serum-free medium to achieve desired concentration, and incubated for another 48 hrs. Cells lysed and luciferase activity analyzed. Cell viability was measured using Neutral Red Method. Data are presented as average fold of control plus/minus standard deviation (n = 3). Open squares represent cell viability, and solid squares luciferase activity.

Figure 8b

Dose-Response of Retinoic Acids in Activating PPRE-aP2 Reporter and Cell Viability in MCF-7 Cells (48 hrs)



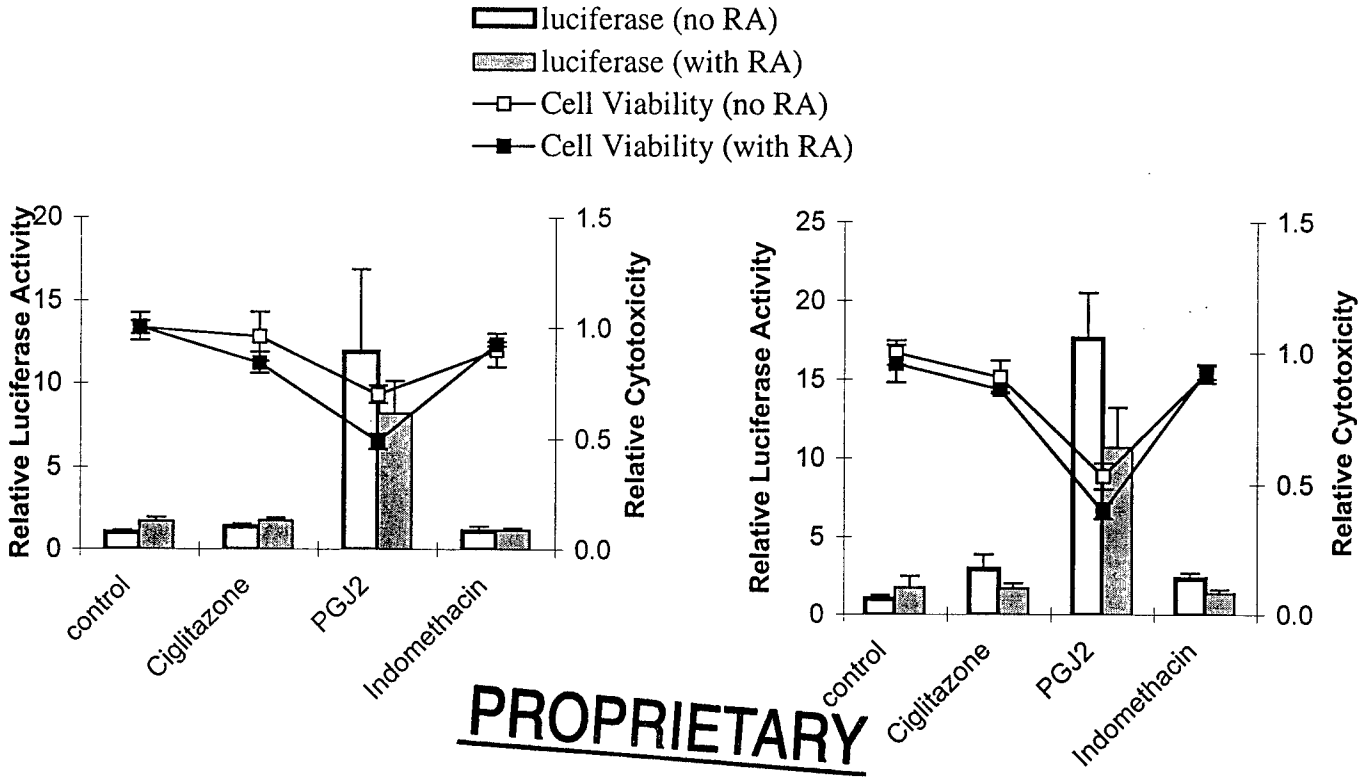
Exponentially grown MCF-7 cells were transiently transfected with either PPRE-aP2-luc reporter alone (left panel) or a mix of PPRE-aP2-luc reporter and rat PPARgamma expression vector (right panel), plated at the density of 20,000/well in 100 ul culture medium containing 10% fetal calf serum into 96-well plates. After incubation at 37 degree for twenty hours, the inducing agents were added in 100 ul serum-free medium to achieve desired concentration, and incubated for another 48 hrs. Cells lysed and luciferase activity analyzed. Cell viability was measured using Neutral Red Method. Data are presented as average fold of control plus/minus standard deviation (n = 3). Open squares represent cell viability, and solid squares luciferase activity.

Figure 8c

Activation of PPRE-aP2 Reporter and Impact on Cell Viability by PPARgamma Ligands in the Presence and Absence of Retinoic Acids in MCF-7 Cells (48 hrs)

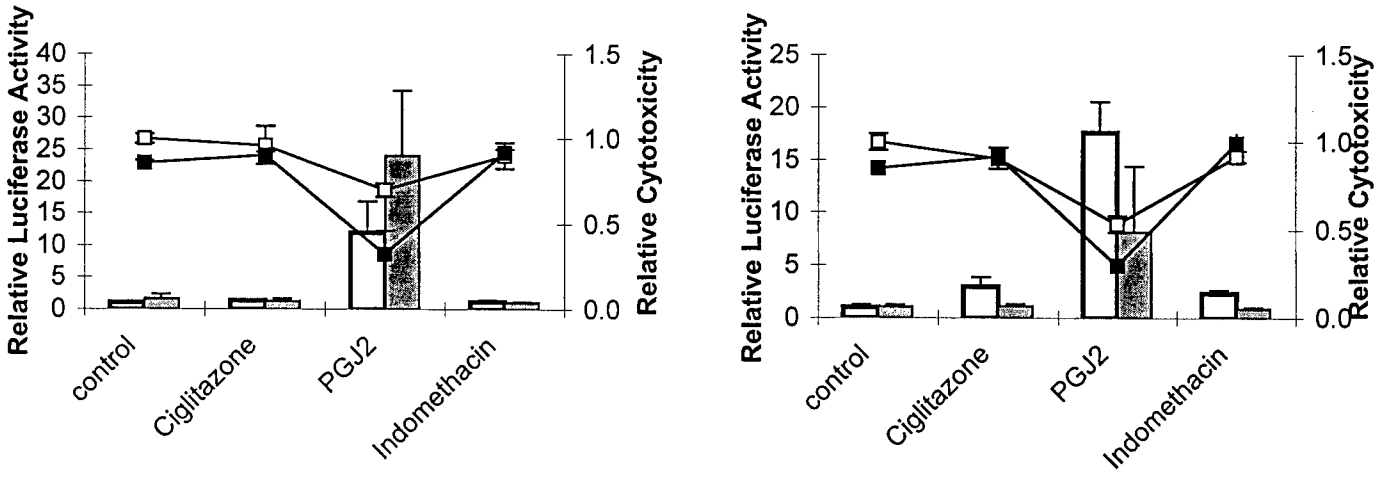
PPRE-aP2-luc Reporter

PPRE-aP2-luc Reporter and rPPARgamma Expression Vector



9-cis-Retinoic Acid

9-cis-Retinoic Acid



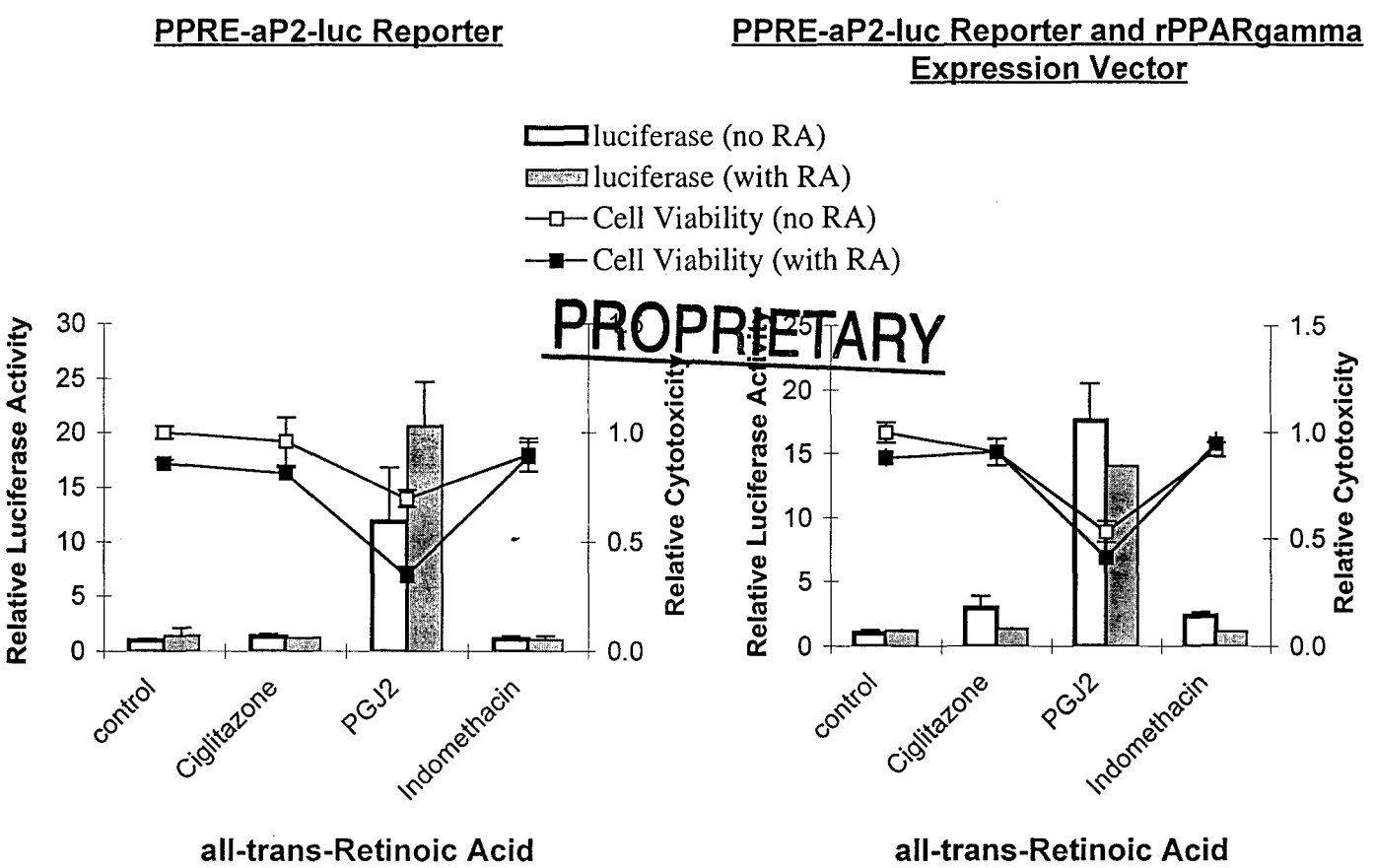
13-cis-Retinoic Acid

13-cis-Retinoic Acid

***Final concentration for all compounds is 10 uM.**

Figure 8d

Activation of PPRE-aP2 Reporter and Impact on Cell Viability by PPARgamma Ligands in the Presence and Absence of Retinoic Acids in MCF-7 Cells (48 hrs)



Exponentially grown MCF-7 cells were transiently transfected with either PPRE-aP2-luc reporter alone (left panel) or a mix of PPRE-aP2-luc reporter and rat PPARgamma expression vector (right panel), plated at the density of 20,000/well in 100 ul culture medium containing 10% fetal calf serum into 96-well plates. After incubation at 37 degree for twenty hours, the inducing agents were added in 100 ul serum-free medium to achieve 10 uM final concentration, and incubated for another 48 hrs. Cells lysed and luciferase activity analyzed. Cell viability was measured using Neutral Red Method. Data are presented as average fold of control plus/minus standard deviation (n = 3). Lines represent cell viability, and solid bars luciferase activity.

Figure 9

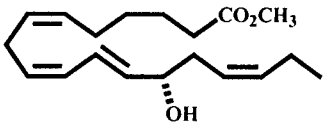
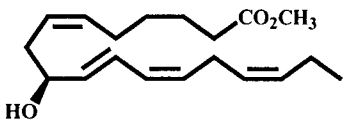
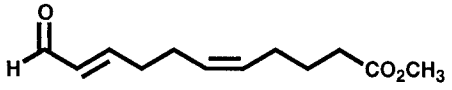
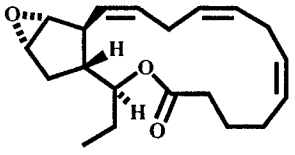
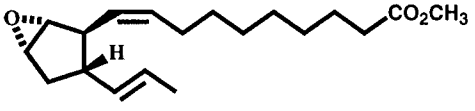
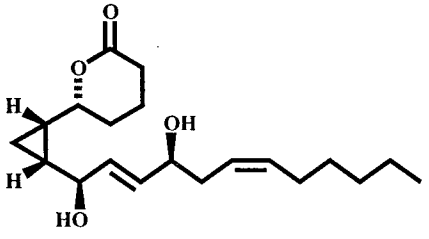
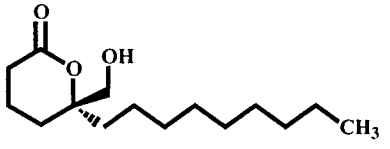
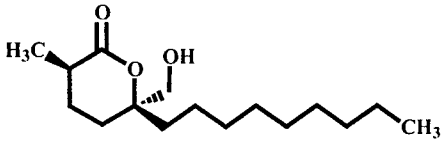
Structure	Compound Name	PPAR-γ Activating Concentration(s) (ppm)
	13(*S*)-HODTE(γ) methyl ester	3.0; 30
	10(*S*)-HODTE(γ) methyl ester	3.0
	cymathere aldehyde - methyl ester	1.0; 3.0
	ecklonialactone A	10.0
	opened ecklonialactone B - methyl ester	3.0
	constanolactone A	1.0
	tanikolide	3.0
	malyngolide	1.0; 3.0, 10

Figure 10

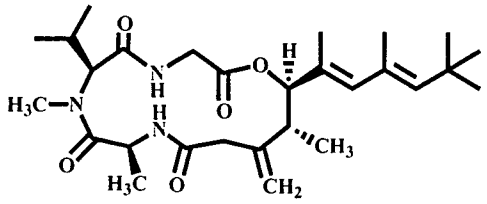
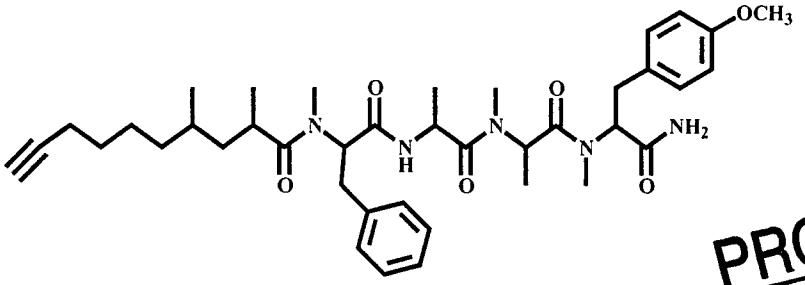
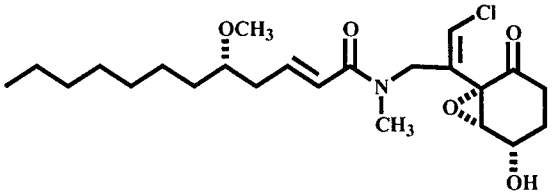
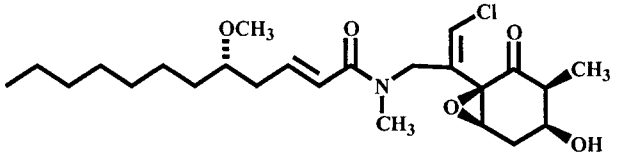
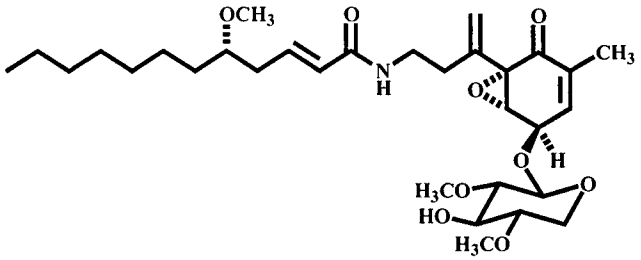
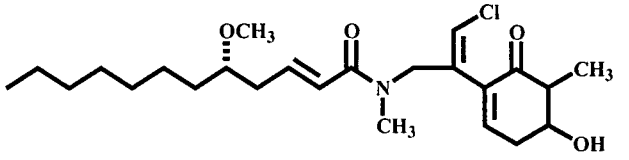
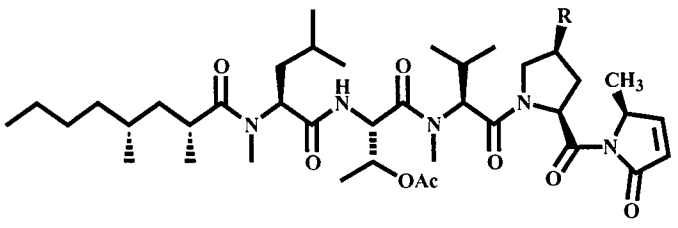
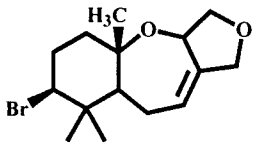
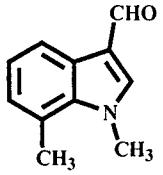
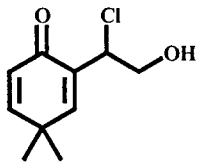
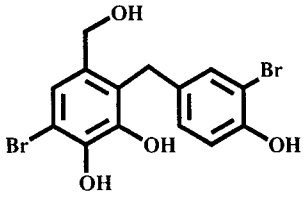
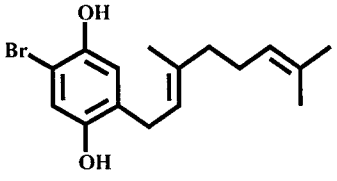
Structure	Compound Name	PPAR-γ Activating Concentration(s) (ppm)
	Antillatoxin	1.0; 3.0
	Carmabin A	1.0
PROPRIETARY		
	Malyngamide C	3.0
	Malyngamide I	1.0; 3.0; 10
	Malyngamide J	3.0
PROPRIETARY		
	Malyngamide L	1.0; 3.0; 10; 30
	Microcolin A: R=OH	1.0
	Microcolin B: R=H	1.0; 3.0; 10

Figure 11

Structure	Compound Name	PPAR-γ Activating Concentration(s) (ppm)
	palisadin A	10
PROPRIETARY		
	*Lyngbya* indole aldehyde	1.0; 3.0; 10
	chlorocyclohexenone	1.0; 3.0; 10
	avrainvilleol	3.0
	cymopol	1.0; 3.0
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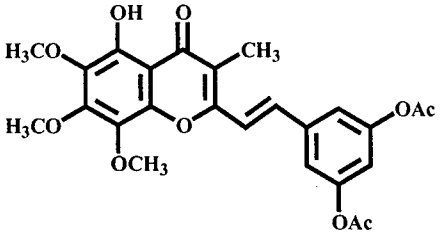	hormothamnione diacetate	3.0

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 28 Aug 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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